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CAPILLARY ELECTROPHORESIS STUDY OF INTERACTION BETWEEN OMEGA-3 FATTY ACIDS AND EPIGALLOCATECHIN GALLATE

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Capillary Electrophoresis Study of Interaction between Omega-3 Fatty Acids and Epigallocatechin Gallate

by

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ABSTRACT

Cancer, a global disease affecting millions of people, is characterized by uncontrolled cell division due to mutations within the cell. The resulting cell mass continues to rapidly grow, resulting in tumours. Epigallocatechin gallate is a polyphenolic catechin found in green tea leaves that has been shown to have antioxidant and anti-inflammatory properties. Recently, it has also been shown to have anti-cancerous effects on various breast cancer cell lines. The effects include inhibiting cancer cell line growth, down-regulating estrogen receptor function, inducing apoptosis, inhibiting tumour promotion, and exhibiting antioxidant properties. This shows that epigallocatechin gallate is a potential cancer therapeutic agent. Although these are promising *in vitro* results, epigallocatechin gallate does not readily cross the cellular phospholipid bilayer *in vivo*. It is hypothesized that epigallocatechin gallate could be carried through the plasma membrane and into the cell when attached to a lipid molecule. The molecule that is currently being tested as a carrier molecule is the omega-3 fatty acid docosahexaenoic acid. The analytical technique affinity capillary electrophoresis has been used to determine that epigallocatechin gallate and docosahexaenoic acid have a reasonably strong interaction of 4.0 ($\pm$ 1.2) x 10^4 at physiological pH.

Thesis Supervisor: Associate Professor Dr. Kingsley Donkor

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1 INTRODUCTION

The health benefits of green tea are becoming more and more prominent in today's society. The numerous benefits that are typically advertised include lowering cholesterol, inhibiting the increase of blood pressure and preventing plaque formation that leads to cavities (the tea spot 2016). Green tea has also been shown to contain high levels of antioxidant and anti-inflammatory properties with loose-leaf green teas having the most benefit (Fujiki 2005). The class of molecules responsible for these vast and favourable health benefits are green tea catechins. Catechins account for 30-40% of the dry weight of solids in steeped green teas and of the four major polyphenolic catechins present in green tea, EGCG, a flaven-3-ol polyphenol, is the most abundant and biologically active (Ishii et al. 2008; Ranzato et al. 2014; Zeng et al. 2014).

Epigallocatechin gallate (EGCG), shown in Figure 1, has been reported to contain many anti-cancerous effects. It prevents cancer formation by exhibiting antioxidant properties such as neutralizing radical oxygen species before they induce cell mutations that lead to tumours (Ishii et al. 2007). It also down-regulates estrogen receptor alpha function that normally controls the growth of breast cancers (Lee et al. 2012). EGCG has also been shown to induce apoptosis of cancerous cells and inhibit tumour promotion (Fujiki 2005; Farabegoli et al. 2007; Ishii et al. 2008). Because of these many anti-cancerous effects, ECGC has been determined to be a possible therapeutic agent for cancer treatments.

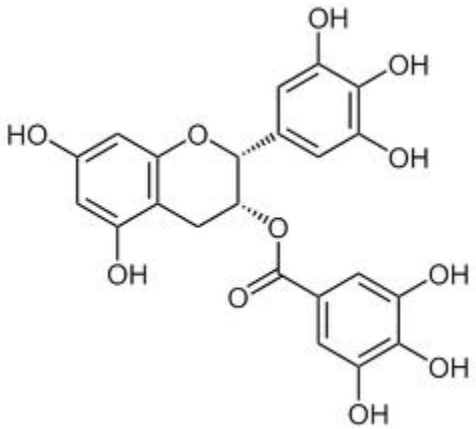

Figure 1. The chemical structure of epigallocatechin gallate, a green tea catechin that has been shown to exhibit antioxidant properties, down-regulate estrogen receptor alpha function, induce apoptosis and inhibit tumour promotion (Farabegoli et al. 2007; Feng 2006; Fujiki 2005; Ishii et al. 2008; Lee et al. 2012).

Cancer is a global disease affecting millions of people on a daily basis. It is characterized as uncontrolled cell division due to mutations within the cell (National Cancer Institute 2016). The resulting cell mass then continues to rapidly grow resulting in tumours. Of all cancers affecting women, breast cancer is the most common in developed countries (excluding non-melanoma skin cancers). It is also the second leading cause of death from cancer in Canadian women (Canadian Cancer Society 2016). Recent research aims to identify new therapeutic possibilities for breast cancer that includes using EGCG.

Zeng and colleagues (2014) investigated the effects of therapeutic EGCG on various breast cancer cell lines. They determined that physiological concentrations of EGCG inhibited growth in estrogen receptor (ER) positive MCF-7 cells by increasing regulation of tumour suppressing genes p53/p21. It also significantly increased cell death in the aggressive ER negative cell line MDA-MB 231. Therapeutics with a potential of targeting MDA-MB 231 cell lines are extremely important as this cell line is extremely aggressive, has poor prognosis and does not respond to common therapies due to its triple negative characteristic (Holliday and Speirs 2011).

Other research conducted by Ranzato and colleagues (2014) also used EGCG as a therapeutic agent with the breast carcinoma cell line MCF-7. Calcium is essential in cell survival, proliferation and growth and disruption of intracellular calcium normally leads to cell death. They showed that EGCG induces a [Ca^{2+}] spike by targeting Ca^{2+} channels, thus suggesting that EGCG deregulates [Ca^{2+}] and leads to decreased MCF-7 viability (Ranzato et al. 2014). Although these *in vitro* results are promising, EGCG is not readily absorbed into the body since it cannot easily cross the phospholipid bilayer (Epstein 2009). However, the therapeutic properties of this catechin may be applicable to humans if it was carried through the plasma membrane via a carrier molecule. Carrier molecules that would be considered are molecules that can easily cross the phospholipid bilayer and enter the cancerous cells. These include lipid molecules such as various omega-3 fatty acids.

Omega-3 fatty acids are essential nutrients that can be found in many dietary foods such as fish oils, flax seeds, eggs, milk and cheese. The body readily absorbs them because they are not endogenously produced (Scarsi et al. 2015). This is an advantage of omega-3 fatty acids as opposed to other potential lipid carriers. Docosahexaenoic acid (DHA) is an omega-3 fatty acid that has a structure of 22:6n-3, shown in Figure 2. DHA can be obtained by eating cold-water fish and from ingesting fish oil supplements. DHA is vital for the growth and cognitive function of the adult brain and is absolutely essential in the brain development of children and is obtained by breast

milk (Horrocks and Yeo 1999). Dietary DHA appears to reduce the risk of heart disease, arthritis, and cognitive and behavioural diseases (Kiruthika 2015; Crauste 2016). Structurally, DHA is a highly unsaturated derivative of alpha-linolenic acid (ALA) (Bougnoux et al. 2010). Although DHA is an ALA derivative, it is not easily converted to DHA in the body; therefore, it must be ingested separately by consuming cold-water fish and fish oil supplements (Horrocks and Yeo 1999; Kiruthika 2015).

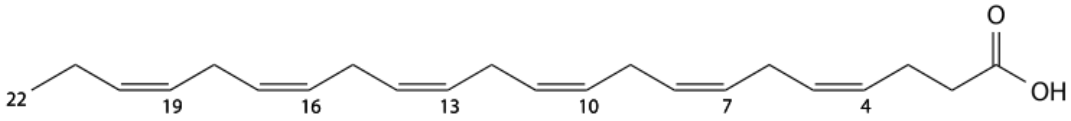

Figure 2. The chemical structure of docosahexaenoic acid, an omega-3 fatty acid that is readily obtained in the diet from cold-water fish (Scarsi et al. 2015).

Recently, the effectiveness of DHA as an addition to common cancer treatments has been studied, specifically in breast cancer treatments. It has been shown to have the potential to increase cancer treatment efficience without serious or aversive side effects (Bougnoux et al. 2010). Research conducted by Hardman and colleagues (2001) determined that rats with xenographs of the triple negative breast cancer cell line MDA-MB 231 were more susceptible to anti-cancer agents that resulted in tumour shrinkage when supplemented with 0.7 grams of DHA on a daily basis (Hardman et al. 2001). These results show that diet may influence or play a significant role in the effectiveness of cancer treatment.

Overall, EGCG and DHA, and their therapeutic effects on breast cancer, have been studied separately, however there is very little in the literature about their interactions. Therefore, by studying the interaction of this EGCG with DHA, we may be able to determine a method that would allow EGCG to have increased permeability to the plasma membrane and thus increase the subsequent effects as a cancer therapeutic agent. An efficient, cheap and time effective analytical method for studying the interaction of these two molecules is capillary electrophoresis.

Capillary electrophoresis (CE) is an analytical method that differentiates and separates charged species on the basis of mobility under the influence of an electric field gradient (Chu et al. 1995). When voltage is applied, electroosmotic flow (EOF) is generated through the buffer solution along the capillary. The sample is then injected into the buffer and is separated and

analyzed based on size and charge. Therefore, small positive ions are detected before small negatively charged ions. This process can be seen below in Figure 3.

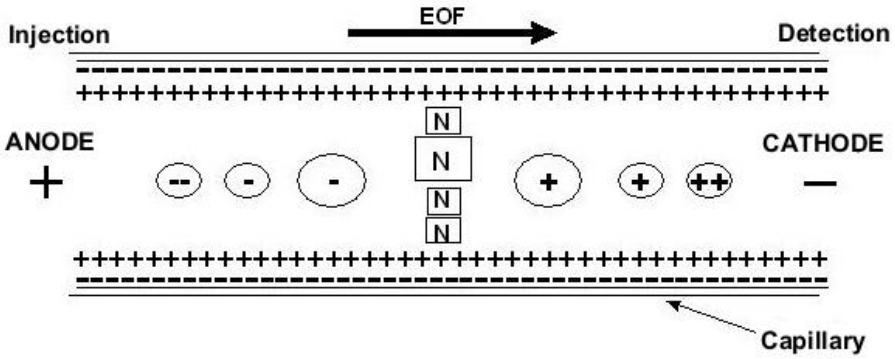

Figure 3. A diagram depicting EOF including the separation of ions based on size and charge between the anode and cathode (Laboratoire Suisse d'Analyse du Dopage 2008).

Non-covalent molecular interactions, such as the suspected interaction of EGCG and DHA, can be quantified using a sub-category method of CE called affinity capillary electrophoresis (ACE). In ACE, the electrophoretic mobility is measured as a function of concentration that a ligand binds the analyte in the electrophoresis buffer (Chu et al. 1995). As the molecules interact inside the capillary, a complex is formed which results in slower migration throughout the capillary and a longer migration time to the detector. The binding constant can then be estimated by the changes in the migration time of the analyte alone compared to the migration time of the complex. In order to determine the change in migration time, a neutral marker is also needed. This compound has no effect on the binding affinity and serves as a reference point when calculating the effective electrophoretic mobility is used to generate the binding constant of EGCG and DHA. The formula for the effective electrophoretic mobility can be seen below in Figure 4.

$$\mu_e = \left[\frac{L_t L_d}{V}\left(\frac{1}{t_c} - \frac{1}{t_m}\right)\right]\left(\frac{I}{I_o}\right)$$

L_t: total length of capillary
L_d: effective length of capillary (to detector)
V: applied voltage
t_c: migration time of complex
t_m: migration time of neutral marker
I: measured current at zero additive concentration
I_o: measured current at given additive concentration

Figure 4. The formula and constants for the effective electrophoretic mobility that is used to quantify binding interactions (Harris 2010).

In this experiment, the capillary will be filled with buffer solution containing varying amounts of DHA with a sample solution at a constant concentration of EGCG. It is expected to see larger EGCG peak shifts with increasing concentrations of DHA. The binding constant can then be estimated by the changes in migration time of EGCG as a function of DHA. This interaction study will be optimized for physiological conditions in order to infer the results to possible therapeutic approaches for breast cancer.

2 MATERIALS AND METHODS

2.1 Reagents

The EGCG standard, the DHA standard, dimethylformamide (DMF) and sodium tetraborate were all purchased from Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada and were all of analytical-reagent grade. The pH of the sodium borate buffers and background electrolyte (BGE) solutions were adjusted by using 1.0 M hydrochloric acid (HCl) and 1.0 M of sodium hydroxide (NaOH). All solutions were prepared using filtered 18 MΩ water and were filtered once again using a 0.45 μm nylon filter before use in the instrument.

2.2 Capillary Electrophoresis

All of the experiments were performed using a Beckman P/ACE™ MDQ Capillary Electrophoresis System (Beckman Coulter, Inc., Fullerton, CA) equipped with an ultraviolet (UV) detector and interfaced to a shared lab computer. Uncoated fused-silica capillary (50 μm internal diameter, 50 cm total length, 40 cm effective length) was used and purchased from Polymicro Technologies (Pheonix, AZ). Galden® HT-110 perfluoropolyether coolant (Ideal Vacuum Products LLC, Albuquerque, NM, USA) was used in the capillary cartridge to keep the temperature at a consistent 25 °C. Detection was set at a wavelength of 214 nm. This wavelength is within the ultraviolet range, which is absorbed by the conjugated double bonds of the EGCG molecule and, to a much lesser degree, by the excess pi electrons in the DHA molecule.

Initially, the capillary was conditioned by flushing 1.0 NaOH through the capillary for 60 minutes, 0.1 M NaOH for 30 min and 18 MΩ water for 10 min. Prior to the sample injection of each run, the capillary was rinsed for 5 minutes with 0.1 M NaOH, 18 MOhm water for 1 min and the run buffer for 4 min. The sample solutions were then injected into the capillary for 5.0 seconds with a pressure of 1.0 psi. Separation of the samples occurred under normal polarity at an applied

voltage of 10 kV. Each run was allowed to separate for 20 min, with the total time for each run being 30 min.

2.3 Preparation of the EGCG and DHA Stock Solutions

A 1000 ppm stock solution of EGCG was prepared by dissolving 5 mg in 5 mL of filtered 18 MΩ water. The stock solution was placed in a glass vial and stored in the fridge at 4^{o}C. A 1000 ppm stock solution of DHA was also prepared by combining 53 μL DHA into 50 mL of filtered methanol. The DHA stock solution was transferred to a brown glass container, as the solution is light sensitive. It was also stored in the fridge at 4^{o}C. All stock solutions were filtered again using a 0.45 μm nylon filter prior to transfer into their storage containers.

2.4 Preparation of Buffer and Background Electrolyte Solutions

A 100 mM stock solution of sodium borate buffer was prepared by dissolving an appropriate amount of sodium tetraborate into filtered 18 MΩ water. The pH of each buffer solution was then adjusted using a pH reader and either 1.0 M HCl or 1.0 M NaOH. A total of three buffer solutions were made with pH's of 9.0, 8.5 and 8.0 and one BGE solution was made with a pH of 7.5.

2.5 Preparation of Run Buffer and EGCG Samples with DMF

The 100 mM borate buffer solution was subsequently diluted to 25 mM borate buffer solution by transferring 250 μL into the CE vial. Filtered 18 MΩ water was then added to each vial to dilute the buffer solution. Various amounts of the 1000 ppm DHA stock solution (0-250 μL) were then added to the vials to equal 0-250 ppm. Each CE vial had a total of 1 mL and were vortexed well. For the samples, 40 μL of the 1000 ppm EGCG stock solution were added to each sample vial, along with 5 μL of a 1:50 DMF solution. Filtered 18 MΩ water was then added to each vial to equal 400 μL and each sample was vortexed well.

3 RESULTS AND DISCUSSION

Prior to conducting the interaction study between EGCG and DHA, many instrumental and experimental parameters had to be optimized. These conditions included the sodium borate buffer and BGE concentration, the concentration of EGCG and the neutral marker.

3.1 EGCG Concentration Optimization

First, the optimal concentration of EGCG was determined. This concentration is important, as it will form a complex with the DHA present in the BGE solution. In this experiment, various concentrations of EGCG (50-150 ppm) were analyzed at a 50 mM BGE solution, as seen below in Figure 5. This work was conducted prior to buffer optimization and the concentration was chosen based on previous studies. A 1 μL aliquot of DMF was added to each sample vial prior to running. The EGCG peaks appear much smaller than the neutral marker peak because of the large quantity of neutral marker added to the EGCG sample. Based on the observed peaks, 100 ppm was selected as the constant EGCG concentration to use in the interactions studies. This also confirms the concentration of previous studies.

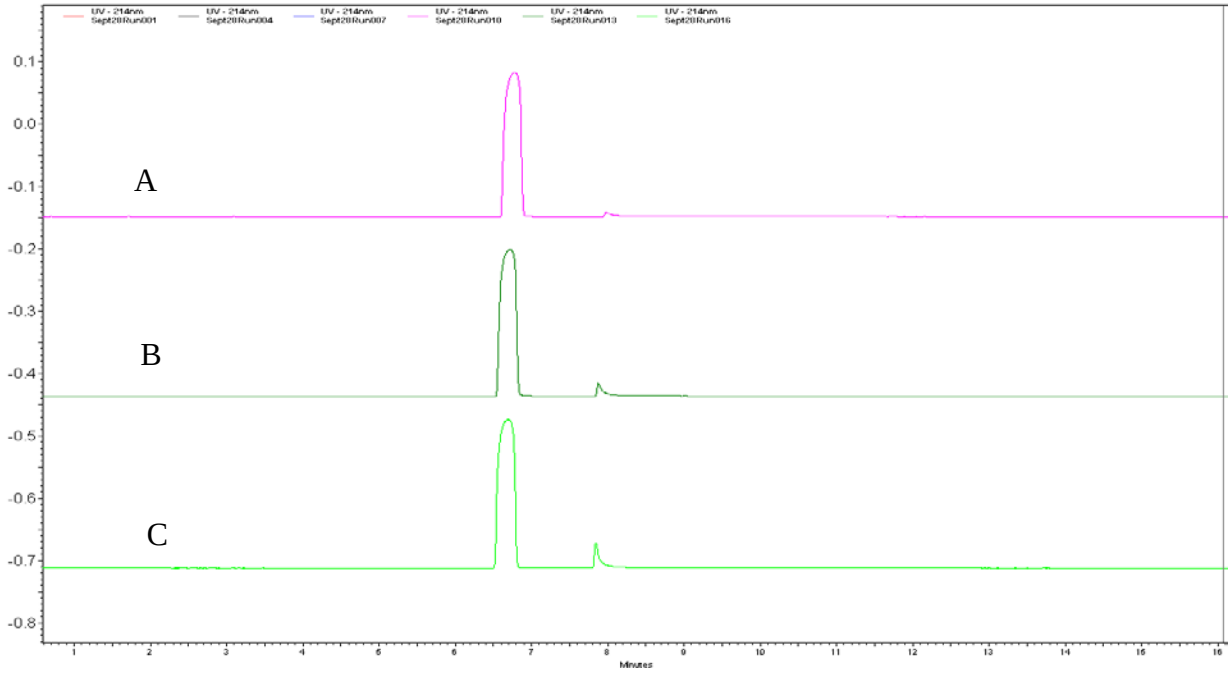

Figure 5. The electropherogram showing various concentrations of EGCG peaks. The top run contained 50 ppm (A), the middle contained 100 ppm (B) and the bottom contained 150 ppm (C). The first peak is the neutral marker (DMF) and the second smaller peak is the EGCG. Conditions: detection, UV; applied voltage, 10 kV; BGE concentration, 50 mM; BGE pH, 7.5; temperature, 25°C.

3.2 Neutral Marker Optimization

The use of a neutral marker is essential when using ACE and serves many purposes. First, it gives an indication of EOF rate and allows for easy observation of any differences of EOF that

may occur across runs. It also serves as a reference point to determine the migration shift of the EGCG and DHA complex peak. Optimization of the amount to add of the neutral marker is important because it should be detectable and proportional to the EGCG peak. Mesityl oxide and DMF were both tested as potential neutral markers. In this experiment, 1 µL of each were added to the EGCG sample vials and their peaks were observed as seen in Figure 6. The height of the mesityl oxide peaks appeared to greatly decrease over the course of the entire experiment. This indicates that it is not very stable and therefore was not chosen for further experiments. DMF, however, showed excellent peak heights consistent over the entire run. Therefore, DMF was used in the following experiments. For these experiments, a 5 µL aliquot of a 1:50 DMF dilution was used in order to obtain appropriate peak heights relative to the EGCG peak.

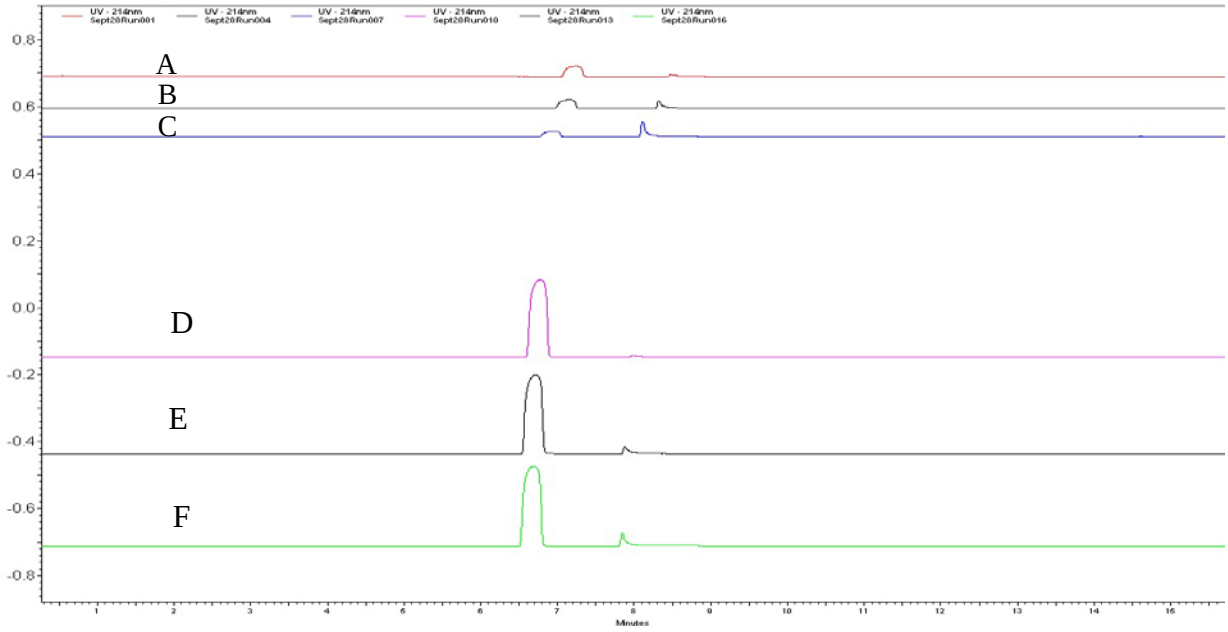

Figure 6. The electropherogram that compares the two potential neutral markers. The top three peaks consisted of a 1 µL aliquot added to samples containing 50 ppm (A), 100 ppm (B) and 150 ppm (C) of EGCG. The bottom three peaks consisted of a 1 µL aliquot also added to samples containing 50 ppm (D), 100 ppm (E) and 150 ppm (F) of EGCG. Based on this data, DMF was used as the neutral marker for the following interaction studies. Conditions: detection, UV; applied voltage, 10 kV; BGE concentration, 50 mM; BGE pH, 7.5; temperature, 25°C.

3.3 BGE Concentration Optimization

The BGE concentration is a very important factor when using CE. It affects the EOF, and can also influence the degree of analyte separate and detection. Initially, four different

concentrations were tested in order to determine the concentration that gave the best EGCG peak shape and baseline separation. These concentrations included 25 mM, 50 mM, 75 mM and 100 mM. The optimal BGE concentration was tested at pH 7.5 in order to apply to physiological pH. As seen below in Figure 7, the optimal BGE concentration appeared to occur at 25 mM. This concentration had the shortest separation time between the neutral marker and the EGCG peaks, which is beneficial because once the EGCG complexes with the DHA, it will migrate further from the neutral marker. If the baseline separation between these two peaks is large without the addition of DHA, then the run time would have to be greatly increased. The 25 mM concentration also gave the best EGCG peak shape compared to the other concentrations. The EGCG peak can be seen to decrease in sharpness and height as the concentration increases until it completely disappears in the 100 mM concentration.

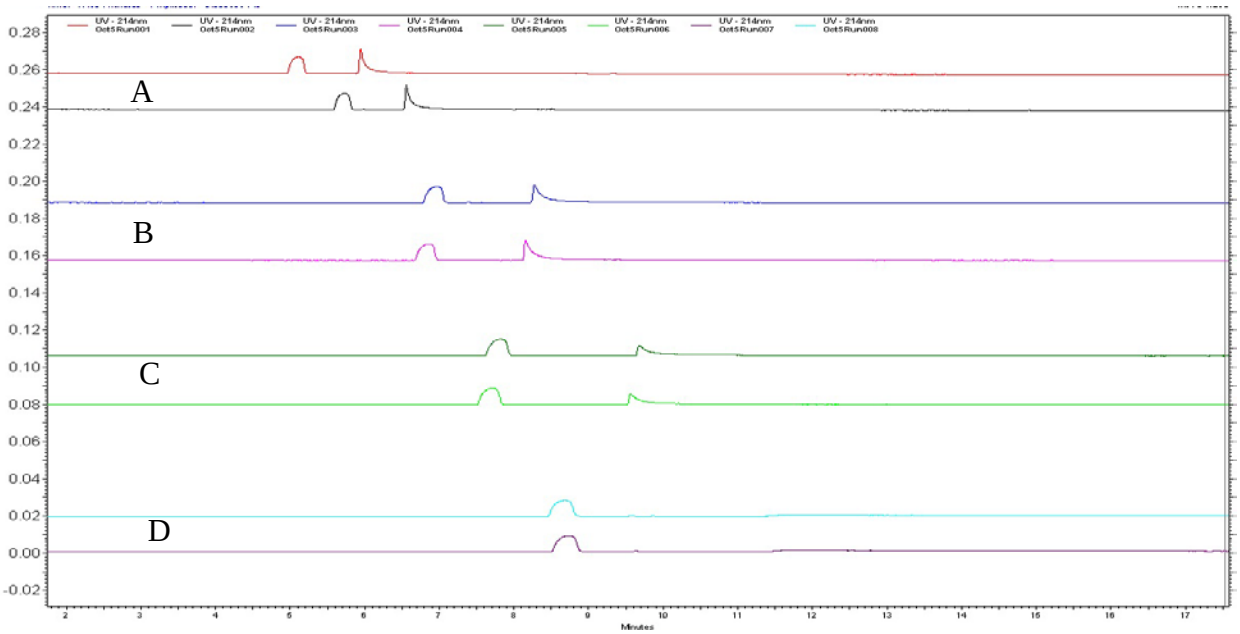

Figure 7. The electropherogram displaying the various BGE concentrations at pH 7.5. Each run was run in duplicates at the concentrations of 25 mM (A), 50 mM (B), 75 mM (C) and 100 mM (D). The first peak is the neutral marker (DMF) and the second peak is the EGCG (100 ppm). Based on the obtained results, 25 mM appeared to give the best EGCG peak shape and baseline separation. Therefore, this concentration was used for all following interaction studies. Conditions: detection, UV; applied voltage, 10 kV; BGE pH, 7.5; temperature, 25ºC.

3.4 Interaction Studies

Once the experimental conditions were optimized, experiments that determined the interaction between EGCG and DHA were conducted. Because the goal of this work is to observe the interaction mimicking physiological conditions, studying the interaction at physiological pH was essential. A similar trend is seen in all of the following interaction studies – as the concentration of DHA increases in the migration of the EGCG complex peak increases as well. This shows that a complex is being formed between EGCG and DHA. The changes in migration can then be used to calculate the binding constant between these two molecules. Another trend that is seen in every interaction study is a slight migration of the neutral marker peak. This occurs due to an increase of viscosity in the BGE solution as the concentration of DHA increases. This migration can be corrected for in the binding constant calculations.

3.4.1 Interaction Study at pH 9.0

The first interaction study was run with a BGE solution of pH 9.0. This pH was chosen because it is an optimal pH to use with a sodium borate buffer and similar work has previously been successful at this pH. The results of the interaction study can be seen in Figure 8.

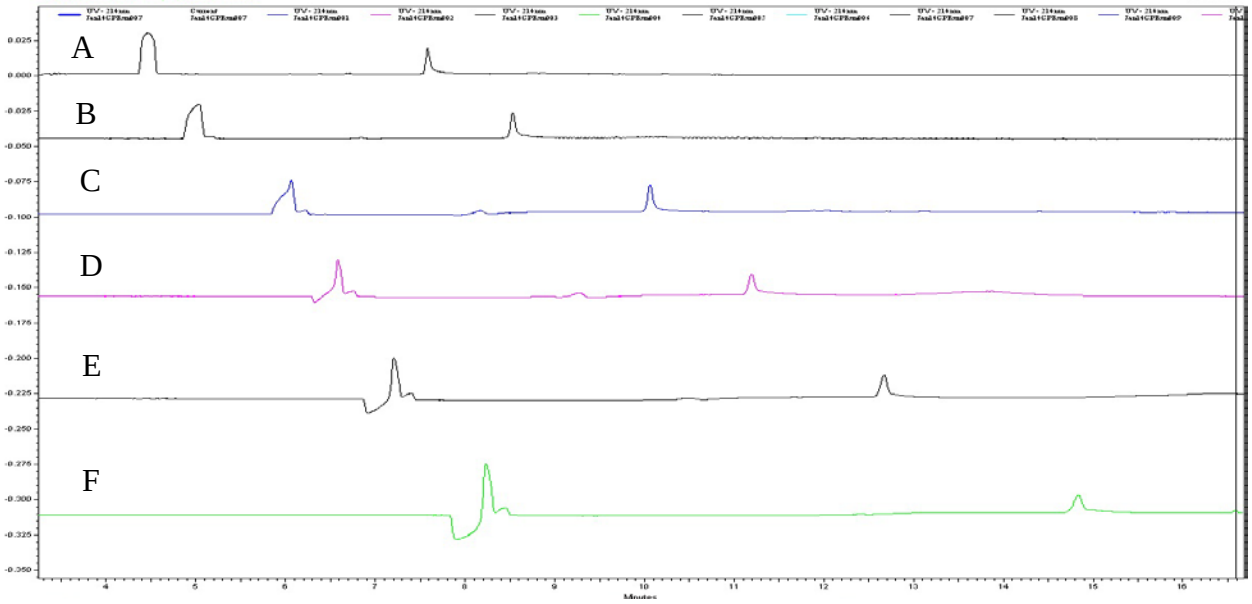

Figure 8. The electropherogram of the interaction study between EGCG and DHA with a 25 mM BGE solution of pH 9.0. The sample consisted of a 5 μL aliquot of a 1:50 DMF dilution as well as a constant concentration of 100 ppm EGCG. The 25 mM BGE solution contained various concentrations of DHA: 0 ppm (A), 50 ppm (B), 100 ppm (C), 150 ppm (D), 200 ppm (E), 250 ppm (F). The formation of a complex as the concentration of DHA increases is evident due to the

increase in migration of the EGCG peak. Conditions: detection, UV; applied voltage, 10 kV; BGE concentration, 25 mM; temperature, 25°C.

3.4.2 Interaction Study at pH 8.5

The second interaction study was run with a BGE solution of pH 8.5. The results of the interaction study can be seen below in Figure 9.

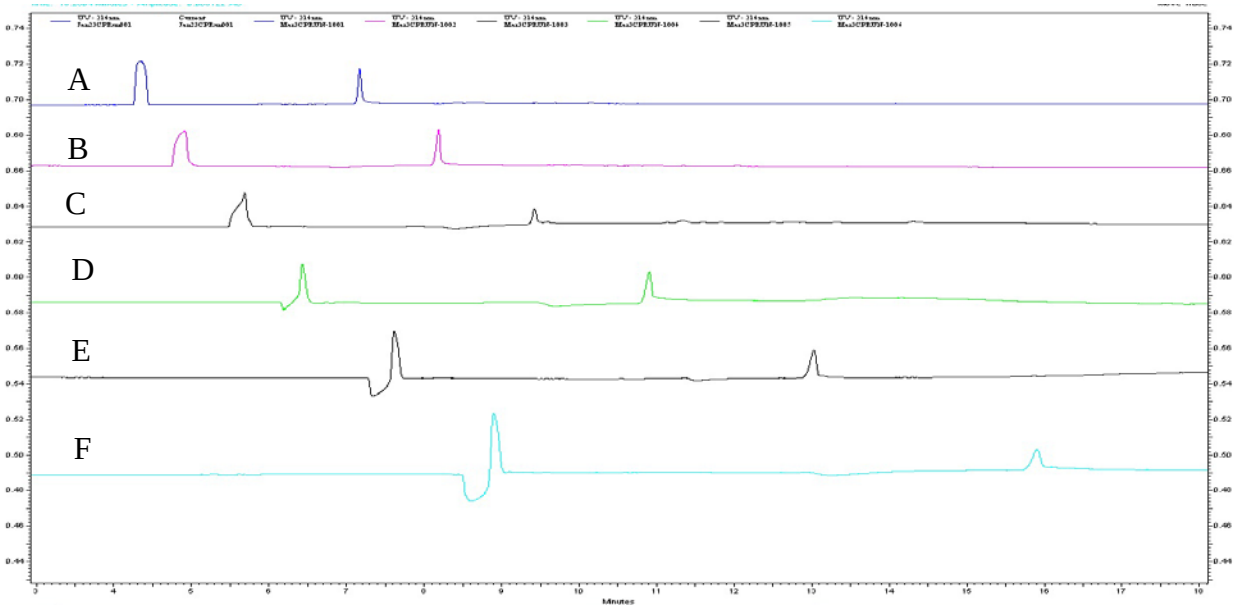

Figure 9. The electropherogram of the interaction study between EGCG and DHA with a 25 mM BGE solution of pH 8.5. The sample consisted of a 5 μL aliquot of a 1:50 DMF dilution as well as a constant concentration of 100 ppm EGCG. The 25 mM BGE solution contained various concentrations of DHA: 0 ppm (A), 50 ppm (B), 100 ppm (C), 150 ppm (D), 200 ppm (E), 250 ppm (F). The formation of a complex as the concentration of DHA increases is evident due to the increase in migration of the EGCG peak. Conditions: detection, UV; applied voltage, 10 kV; BGE concentration, 25 mM; temperature, 25°C.

3.4.3 Interaction Study at pH 8.0

The third interaction study was run at a with a BGE solution of pH 8.0. The results of the interaction study can be seen below in Figure 10.

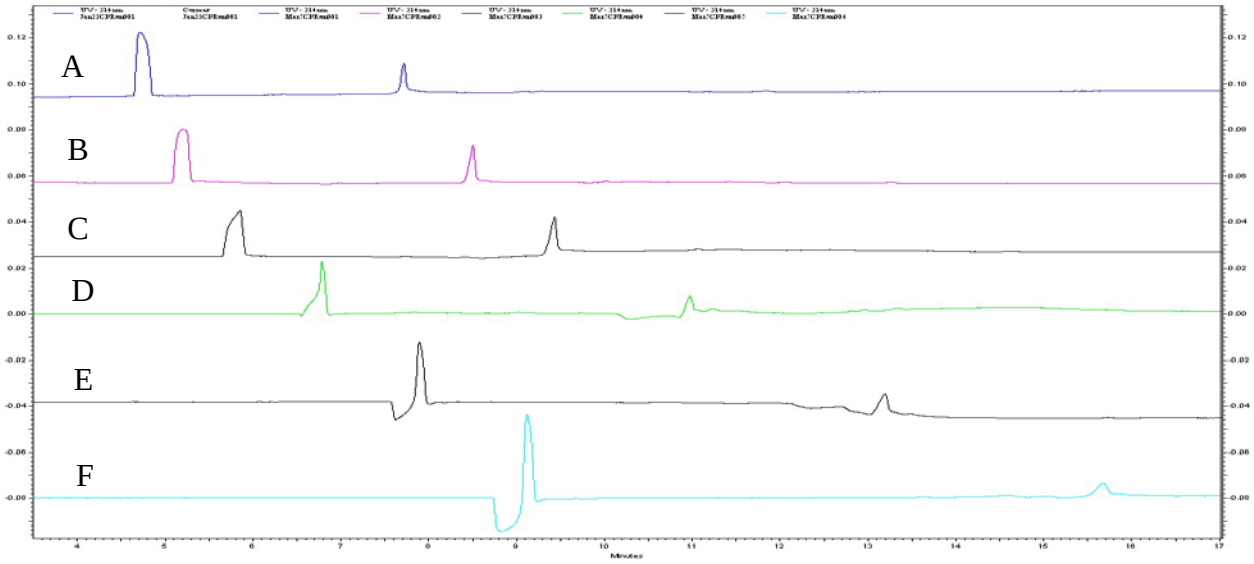

Figure 10. The electropherogram of the interaction study between EGCG and DHA with a 25 mM BGE solution of pH 8.0. The sample consisted of a 5 μL aliquot of a 1:50 DMF dilution as well as a constant concentration of 100 ppm EGCG. The 25 mM BGE solution contained various concentrations of DHA: 0 ppm (A), 50 ppm (B), 100 ppm (C), 150 ppm (D), 200 ppm (E), 250 ppm (F). The formation of a complex as the concentration of DHA increases is evident due to the increase in migration of the EGCG peak. Conditions: detection, UV; applied voltage, 10 kV; BGE concentration, 25 mM; temperature, 25^{o}C.

3.4.4 Interaction Study at pH 7.5

The fourth interaction study was run at a with a BGE solution pH of 7.5. The results of the interaction study can be seen below in Figure 11. Based on these results, an interaction does occur between EGCG and DHA at physiological pH.

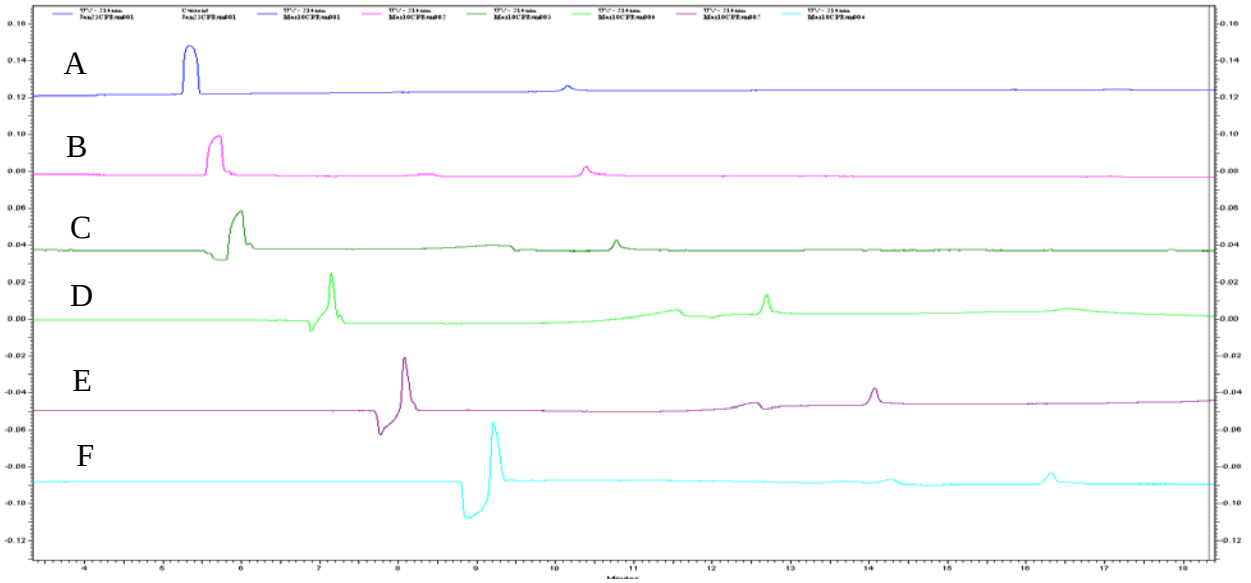

Figure 11. The electropherogram of the interaction study between EGCG and DHA with a 25 mM BGE solution of pH 8.0. The sample consisted of a 5 μL aliquot of a 1:50 DMF dilution as well as a constant concentration of 100 ppm EGCG. The 25 mM BGE solution contained various concentrations of DHA: 0 ppm (A), 50 ppm (B), 100 ppm (C), 150 ppm (D), 200 ppm (E), 250 ppm (F). The formation of a complex as the concentration of DHA increases is evident due to the increase in migration of the EGCG peak. Conditions: detection, UV; applied voltage, 10 kV; BGE concentration, 25 mM; temperature, 25°C.

Based on these results, it appears that an interaction does occur between EGCG and DHA at physiological pH. This study was run in duplicates in order to obtain enough data to derive a significant binding constant.

3.5 Binding Constant

The binding constant between the molecules EGCG and DHA was calculated from the data obtained from the pH 7.5 experiments. It was analyzed using the linear regression method as outlined in detail in the appendix. From this the x-reciprocal, the y-reciprocal and the double reciprocal plots were obtained. The plots had R^2 values of 0.8585, 0.9997 and 0.8548, respectively. From these plots, the equation of the lines were used to calculate the binding constants. The calculations that were used can be found in the appendix. The binding constants from the x-reciprocal, y-reciprocal and double reciprocal were determined to be 4.41×10^4, 4.92×10^4 and 2.65×10^4, respectively. These values gave an average binding constant value of 4.0×10^4 ($\pm 1.2 \times 10^4$), as seen in Table 1. This value implies that there is a reasonably strong interaction between

EGCG and DHA at physiological pH. This value is also very close to previous work that determined the binding constants of fatty acids with beta-cyclodextrin (Parker and Stalcup 2008).

Table 1. The binding constants determined for EGCG and DHA at pH 7.5 that were calculated using linear regression plots.

Plot	**Binding Constant**
X-Reciprocal	4.41×10^4
Y-Reciprocal	4.92×10^4
Double Reciprocal	2.65×10^4
Average	**$4.0 (\pm 1.2) \times 10^4$**

As seen in the appendix, all three linear regression plots resulted in large and significant R^2 values that were greater than 0.85. Because ACE assumes a 1:1 binding system, the binding stoichiometry cannot be confirmed unless further analysis is conducted. However, the benefit of using the linear regression model over other models to derive binding constants is that the binding stoichiometry can be inferred from the x-reciprocal plot. A linear plot is indicative of a 1:1 binding stoichiometry compared to a nonlinear plot that may imply a 1:2 binding stoichiometry. In this experiment, I would expect a 1:1 binding stoichiometry of EGCG and DHA based on the x-reciprocal plot.

4 CONCLUSION AND FUTURE WORK

ACE was shown to be a successful technique for studying the interaction between EGCG and DHA at physiological conditions. By using optimized instrumental conditions for ACE (detection, UV; applied voltage, 10 kV; BGE concentration, 25 mM; BGE pH, 7.5; temperature, 25^{o}C), the strength of the interaction between EGCG and DHA could be determined. With these conditions, the migration times for the EGCG peak and the EGCG/DHA complex peak were measured and analyzed. They were analyzed using the linear regression method (outlined in the appendix) that assumes a 1:1 binding system. At physiological pH the binding constant was determined to be 4.0 ($\pm$ 1.2) x 10^4 by using the linear regression method. This binding constant value indicates that a reasonably strong interaction occurs between EGCG and DHA at

physiological pH and 25^{o}C. Also, based on the linear plot obtained from the x-reciprocal plot shown in the appendix, I would assume a 1:1 binding stoichiometry between EGCG and DHA. Therefore, DHA can be selected as a potential carrier molecule that would bind and subsequently transport EGCG through the plasma membrane and into the cancerous cell. More investigation into the specifics of this interaction must be studied in order to determine the potential of this cancer therapeutic strategy.

Future work for this research would be to run the triplicate of the interaction study with a BGE solution of pH 7.5 in order to obtain more statistically significant results. Also, the type of interaction should be determined by conducting a thermodynamic study. Because we want to study this interaction as close to physiological conditions as possible, the temperature the interaction occurs at should be modified to 37^{o}C (the temperature inside the body). The sites of binding should also be investigated because ACE assumes a 1:1 interaction. This can be done either by capillary electrophoresis-frontal analysis or by studying proton nuclear magnetic resonance (^{1}H-NMR). Finally, other Omega-3 fatty acids should be investigated with these instrumental conditions such as ALA.

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6 APPENDIX

6.1 Linear Regression Method for Calculating Binding Constants

During the formation of the EGCG and DHA complex, a rapid equilibrium is occurring that favours the formation of the complex. The complex occurs in a 1:1 stoichiometry that can be seen in Equation 1.

$$\mathrm{EGCG} + \mathrm{DHA} \rightleftharpoons \mathrm{EGCGDHA} \quad (1)$$

The binding constant (K) is then calculated in Equation 2 as follows:

$$\mathrm{K} = \frac{[\mathrm{EGCGDHA}]}{[\mathrm{EGCG}][\mathrm{DHA}]} \quad (2)$$

The electrophoretic mobility (μ_e), discussed in the intro, can then be calculated using Equation 3.

$$\mu_e = \left[\frac{L_t L_d}{V}\left(\frac{1}{t_c} - \frac{1}{t_m}\right)\right]\left(\frac{I_o}{I}\right) \quad (3)$$

Equation 3 can also be given as Equation 4 to determine the electrophoretic mobility of the free analyte, μ_o, given in Equation 5, as well as the electrophoretic mobility of the complex, μ_c in Equation 6.

$$\mu_e = \frac{\mu_o + \mu_c \mathrm{K}[\mathrm{DHA}]}{1 + \mathrm{K}[\mathrm{DHA}]} \quad (4)$$

$$\mu_o = \frac{L_d L_t}{V t_o} \quad (5)$$

$$\mu_c = \frac{L_d L_t}{V t_c} \quad (6)$$

Finally, the electrophoretic mobility equation can be rearranged to give three linear equations. These linear equations are then used to plot the double reciprocal (Equation 7), the y-reciprocal (Equation 8) and the x-reciprocal (Equation 9).

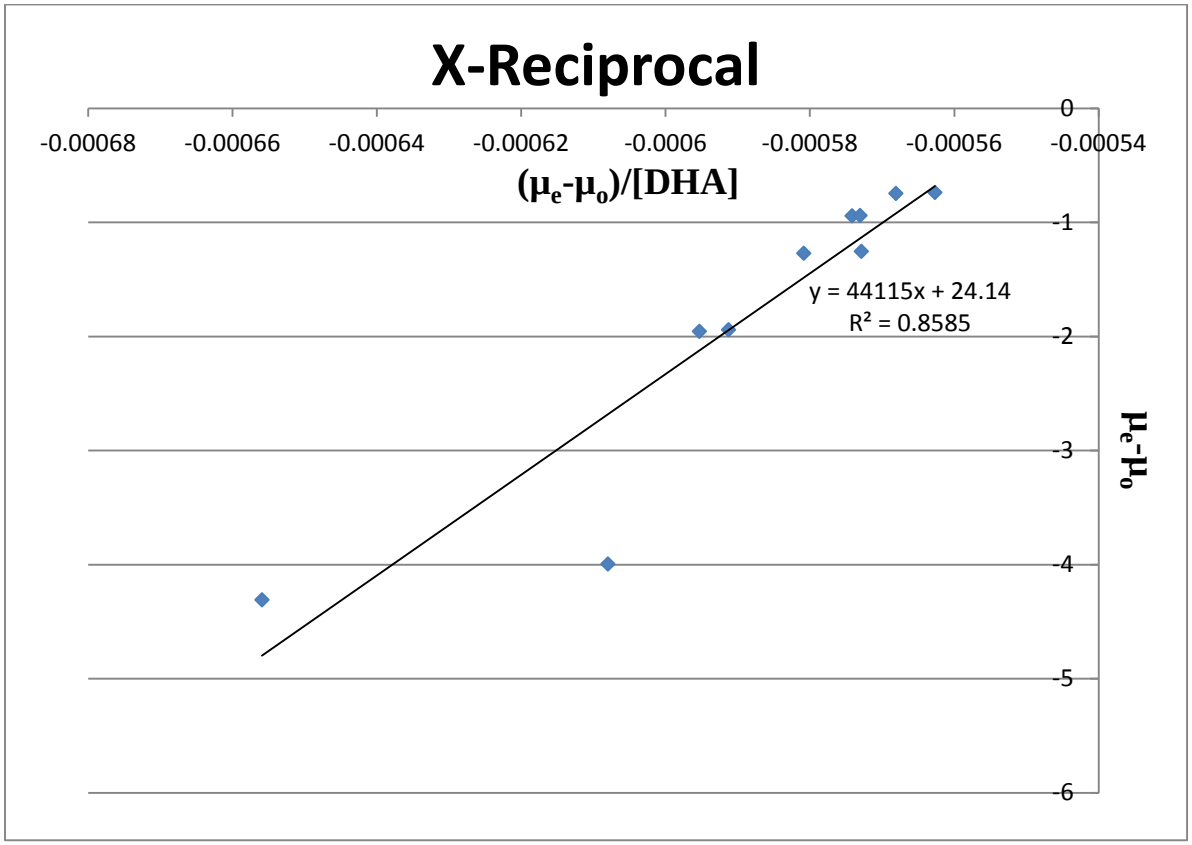
$$\frac{1}{\mu_e - \mu_o} = \frac{1}{(\mu_c - \mu_o)\mathrm{K}} \cdot \frac{1}{\mathrm{DHA}} + \frac{1}{\mu_c - \mu_o} \quad (7)$$

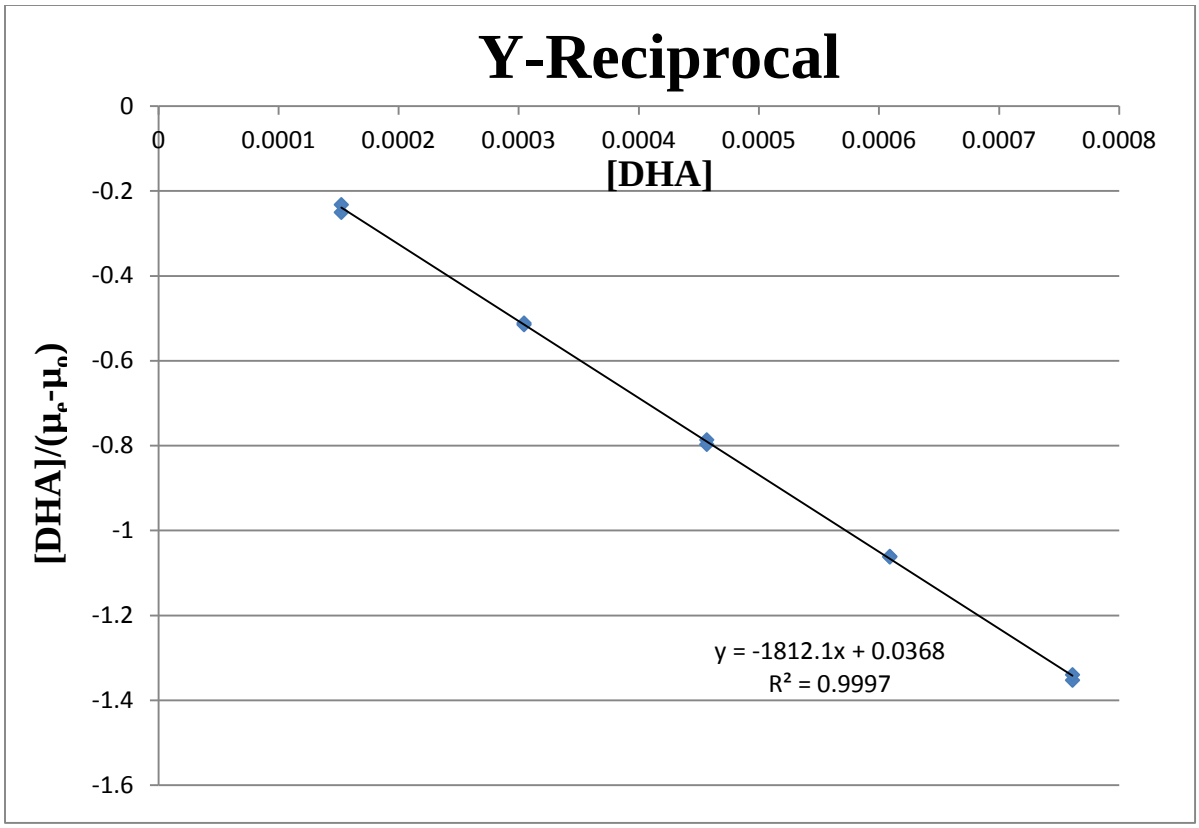
$$\frac{[\mathrm{DHA}]}{\mu_e - \mu_o} = \frac{1}{(\mu_c - \mu_o)} \cdot [\mathrm{DHA}] + \frac{1}{(\mu_c - \mu_o)\mathrm{K}} \quad (8)$$

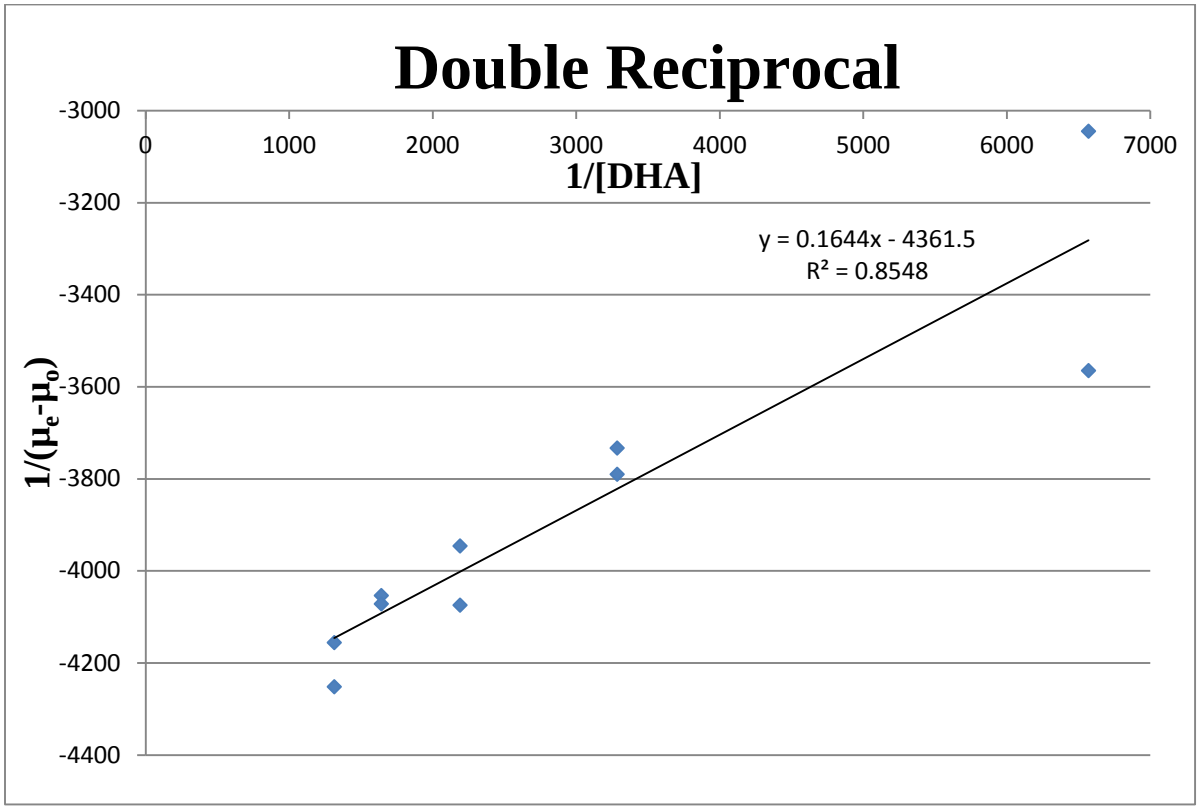
$$\frac{\mu_e - \mu_o}{[\mathrm{DHA}]} = -\mathrm{K}(\mu_e - \mu_o) + \mathrm{K}(\mu_c - \mu_o) \quad (9)$$

For the double reciprocal, the values of $\frac{1}{\mu_e - \mu_o}$ are plotted against $\frac{1}{\mathrm{DHA}}$ and the binding constant is calculated by dividing the y-intercept by the slope of the equation. For the y-reciprocal, the $\frac{[\mathrm{DHA}]}{\mu_e - \mu_o}$ values are plotted against the [DHA]. The y-reciprocal binding constant is then calculated by dividing the slope of the equation by the y-intercept. Lastly, the x-reciprocal is plotted with $\frac{\mu_e - \mu_o}{[\mathrm{DHA}]}$ values against $(\mu_e - \mu_o)$ values. This binding constant is then determined by taking the negative of the slope of the equation. All of the binding constant values can then be averaged to obtain a significant binding constant value.

6.2 Linear Regression Plots




Y-Reciprocal
0
0
0.0001
0.0002
0.0003
0.0004
0.0005
0.0006
0.0007
0.0008
[DHA]
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
-1.6
[DHA]/(μe-μo)
y = -1812.1x + 0.0368
R² = 0.9997


Double Reciprocal
-3000
0
1000
2000
3000
4000
5000
6000
7000
1/[DHA]
-3200
-3400
-3600
-3800
-4000
-4200
-4400
1/(μe-μo)
y = 0.1644x - 4361.5
R² = 0.8548